ICS 67.050
C149

团　　体　　标　　准

T/CASME 012-2021

β-烟酰胺单核苷酸纯度和含量的测定　高效液相色谱法

Determination for β-nicotinamide mononucleotide content　High performance liquid chromatography

（征求意见稿）

2021 -**- **发布　　2021 - **- **实施

中国检验检疫科学研究院综合检测中心
中国中小企业商业协会 发布

版权声明

联系人：乐粉鹏

联系电话：010-85767344

联系邮箱：yuefenpeng@caiqtest.com

前　言

本文件参照 GB/T 1.1-2020《标准化工作导则-第 1 部分：标准化文件的结构和起草规则》给出的规则起草。请注意本文件的某些内容有可能涉及专利，本文件的发布机构不应承担识别这些专利的责任。

本文件由中国检验检疫科学研究院综合检测中心提出，由中国中小商业企业协会归口。

本文件起草单位：

本文件主要起草人：

本文件为首次发布。

β-烟酰胺单核苷酸纯度和含量的测定　高效液相色谱法

1　范围

本文件规定了β-烟酰胺单核苷酸纯度和含量测定方法的术语定义、试剂和材料、仪器设备、标准溶液配制、样品前处理、色谱条件、试样测试和结果计算。

本文件适用于以生物法生产而成的原料和成品中β-烟酰胺单核苷酸纯度和含量的测定方法。

2　术语和定义

2.1　β-烟酰胺单核苷酸

NMN（Nicotinamide mononucleotide）：全称“β-烟酰胺单核苷酸”，是一种自然存在的生物活性核苷酸。NMN有2种存在形式，α和β；β异构体是NMN的活性形式。

2.2　β-烟酰胺单核苷酸分子式、CAS号和分子量

2.2.1 分子式：$C_{11}H_{15}N_2O_8P$

2.2.2 CAS号：1094-61-7

2.2.3 相对分子质量：334.22（按2016年国际相对原子质量）

2.3 生物法

生物法是指以发酵、生物转化、酶法、细胞培养和生物提取为一种或多种组合手段为技术的生产工艺。

3　试剂和材料

以下所用的试剂,除特别注明外均为分析纯试剂。

3.1　β-烟酰胺单核苷酸对照品：含量不低于98%（HPLC）。

3.2　甲醇：色谱级。

3.3　磷酸二氢钾。

3.4　四丁基硫酸氢铵。

3.5　氢氧化钾。

3.6　一级水。

4 仪器设备

4.1 高效液相色谱仪：配有紫外检测器或 DAD 检测器和柱温箱系统。

4.2 色谱柱：C18 ODS-BP 5um 4.6mm x 250mm （或同等分析效果的色谱柱）。

4.3 分析天平：最小分度值 0.01 mg。

4.4 超声波溶解器。

4.5 微孔过滤器。

4.6 微孔滤膜：0.45μm。

4.7 粉碎机。

5 标准溶液配制

5.1 标准储备液的制备 ：1000mg/L

准确称取 100mg（精确至 0.0001g） β-烟酰胺单核苷酸的标准品至 100mL 容量瓶中，用一级水溶解，经超声处理使完全溶解，定容，经 0.45um 水系微孔滤膜过滤，滤液浓度为 1000mg/L 的标准储备液。

5.2 标准中间液的制备 ：250mg/L

准确移取 25mL 标准储备液（A.3.1）于 100mL 容量瓶中，用一级水稀释至刻度，配制成浓度为 250 mg/L 的标准中间液。

5.3 标准工作液的制备

准确移取 0、1、2.5、5、10、25mL 标准储备液（A.3.2）于 25mL 容量瓶中，用一级水稀释至刻度，配制成浓度分别为 0、10、25、50、100、250 mg/L 的标准工作液。

6 样品前处理

6.1 原料样品：

称取 100mg（精确至 0.01 mg）供试品至 100mL 容量瓶中，用一级水溶解，经超声处理使完全溶解，定容，经 0.45um 水系微孔滤膜过滤，滤液为供试品样品溶液。

6.2 成品样品：

6.2.1 每粒成品净含量

随机抽取10粒及以上的成品或其内容物进行称量（精确至0.01 mg），按照所取数量求其平均值，得到每粒成品样品的净含量。

6.2.2 β-烟酰胺单核苷酸提取

称取内容物粉末或磨碎后样品约50mg（精确至0.01 mg）至100mL容量瓶中，加入50mL一级水，超声溶解20min，再用一级水定容至刻度，经0.45um水系微孔滤膜过滤，滤液移入液相进样瓶中，待测。若样品测试浓度超出工作曲线范围，可适当稀释后再测。

7 色谱分析条件

7.1 柱温：30℃。

7.2 检测波长：254nm。

7.3 流动相A：称取13.6g磷酸二氢钾，2.7g四丁基硫酸氢铵溶于800mL一级水中，搅拌至全溶。用氢氧化钾溶液调节pH至3.5±0.05，补水至1000mL，加甲醇64mL，搅拌均匀，最后用0.45um微孔滤膜抽滤，超声脱气即可。

7.4 流动相B：用量筒量取700mL甲醇用一级水定容至1000mL，超声处理后备用。梯度洗脱程序见表1。

7.5 进样量：10μL。

表1 梯度洗脱程序

时间/min	流速/（mL/min）	B%
0	0.8	0
12	0.8	6
17	0.8	70
19	0.8	70
21	0.8	0
30	0.8	0

8 试样测定

8.1 原料样品

在色谱条件下，分别对标准储备液（5.1）和原料样品溶液（6.1 ）进行检测，记录色谱峰的面积和保留时间。根据色谱峰的面积，以面积归一化法计算产品纯度，以单点外标定量法计算产品的含量。

8.2 成品样品

在色谱条件下，分别对标准工作液（5.3）和样品溶液（6.2）进行检测，记录色谱峰的面积和

保留时间。根据色谱峰的面积，以外标法计算产品含量。

9 结果计算

9.1 原料样品

9.1.1 纯度计算

试样中 β-烟酰胺单核苷酸纯度按式(1)计算:

$$P=\frac{A_T}{A_0}\times 100\% \quad \text{.....（1）}$$

式中： P——原料中 β-烟酰胺单核苷酸的纯度，单位为百分比（%）；

A_T——原料样品溶液（6.1）中 β-烟酰胺单核苷酸主峰面积；

A_0——原料样品溶液（6.1）中所有峰的面积和。

9.1.2 含量计算

试样中 β-烟酰胺单核苷酸含量按式(2)计算:

$$X=\frac{C_T\times V}{\mathrm{m}\times 10000} \quad \text{.....（2）}$$

式中：X——供试品中 β-烟酰胺单核苷酸的含量，单位为百分比（%）；

C_T——由单点定量外标法得到原料样品溶液（6.1）中 NMN 的浓度（mg/L）；

V——试样的定容体积（mL）；

m——试样的质量, 单位为克(g)。

9.2 成品样品

9.2.1 每粒成品样品的净含量按式（3）计算

$$W=\frac{\mathrm{m}_{总}}{N} \quad \text{.....（3）}$$

式中：W——每粒成品样品的净含量（g/粒）；

$\mathrm{m}_{总}$——称取所有数量成品的总质量（g）

N——称取所有成品的数量（粒）

9.2.2 试样中 β-烟酰胺单核苷酸含量按式（4）计算:

$$X=\frac{C\times V\times \mathrm{f}}{m\times 1000}\times W \quad \text{.....（4）}$$

式中：X——成品样品中 β-烟酰胺单核苷酸的含量，单位为（mg/粒）；

C ——样品溶液（6.2）中 β-烟酰胺单核苷酸浓度（mg/L）；

V——定容体积（mL）；

f——稀释倍数；

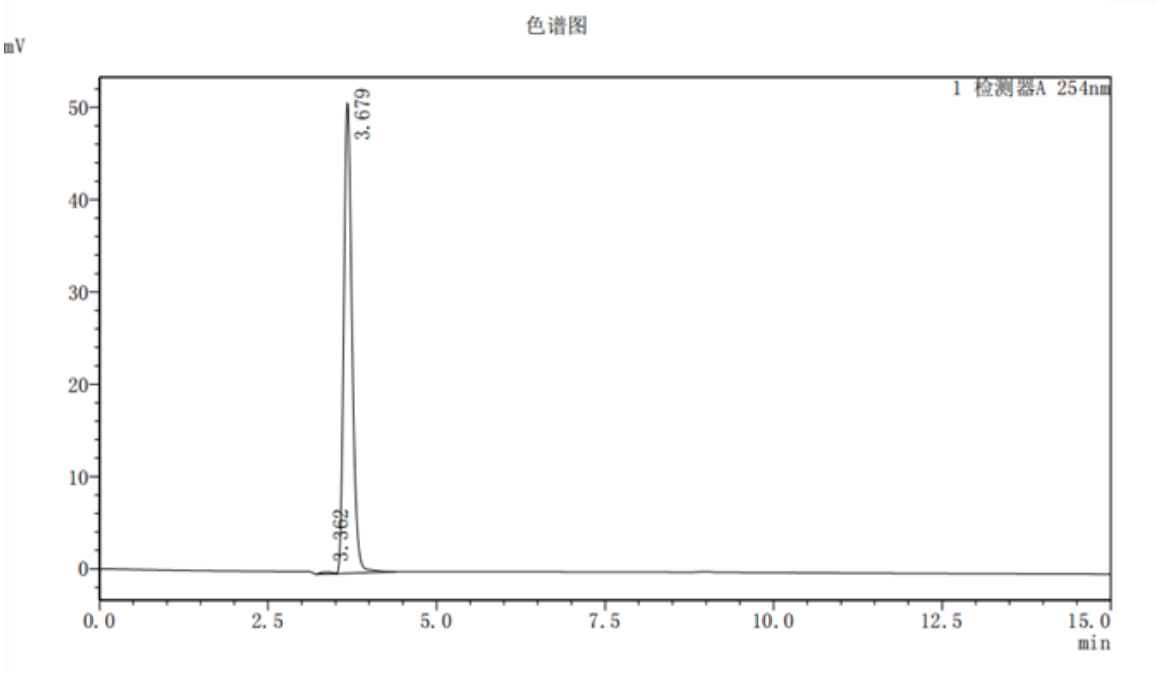
m——成品样品的称样量（g）；

W——每粒成品样品的净含量（g）。

附录 A

（资料性）

图 1 参考 β-烟酰胺单核苷酸液相色谱图



（English Version）

Determination for β-nicotinamide Mononucleotide Content High Performance Liquid Chromatography

1. Scope

This document stipulates the terms and definition, reagents and materials, instruments, standard solution preparation, pre-treatment for samples, chromatograph conditions, sample testing and result calculation of the determination method of β-nicotinamide Mononucleotide.

This document is applicable for the determination method of β-nicotinamide Mononucleotide content in raw materials and finished products produced with biological method.

2. Terms and definition

2.1 β-Nicotinamide Mononucleotide

NMN（Nicotinamide mononucleotide）: The full name is "β-Nicotinamide Mononucleotide"; it is a naturally existed biological active nucleotides, existing in 2 forms of α and β; β isomer is the active form of NMN.

2.2 Molecular formula, CAS NO. and molecular mass of β-Nicotinamide Mononucleotide

2.2.1 Molecular formula: $C_{11}H_{15}N_2O_8P$

2.2.2 CAS NO.: 1094-61-7

2.2.3 Relative molecular mass: 334.22 (as per the international relative atomic mass of 2016)

2.3 biological method

Biological method refers to the production process with fermentation, biotransformation, enzymatic method, cell culture and biological extraction as one or more combination means.

3. Reagents and materials

The below used reagents are all analytical reagents unless otherwise marked.

3.1 Reference product of β-Nicotinamide Mononucleotide: The content is not lower than 95%.

3.2 Methanol: Chromatography grade

3.3 Potassium dihydrogen phosphate

3.4 Tetrabutylammonium hydrogen sulfate

3.5 Potassium hydroxide

3.6 Primary water

4. Instrument

4.1 High performance liquid chromatography: equipped with ultraviolet detector or DAD detector and column heater system.

4.2 Chromatographic column: C18 ODS-BP 5um 4.6mm x 250mm (or chromatographic column with the equivalent analysis effect).

4.3 Analysis balance: with the minimal graduation value as 0.01mg

4.4 Ultrasonic dissolver

4.5 Micropore filter

4.6 Micropore filtering membrane: 0.45μm

4.7 Grinder

5. Preparation of standard solution

5.1 Preparation of standard stock solution：1000mg/L

Accurately weigh 100mg (precise to 0.01mg) standard product of β-Nicotinamide Mononucleotide and dissolve it into a bottle of 100mL completely through ultrasonic treatment, make the volume constant, filter the solution with 0.45um micropore filtering membrane for water system, and concentrate the filtrate into standard stock solution of 1000mg / L.

5.2 Preparation of standard intermediate solution：250mg/L

Accurately weigh 25mL standard stock solution (A.3.1) and dilute it into a bottle of 100mL with primary water to the scale, and prepare it into standard intermediate solution with the concentration as 250mg / L.

5.3 Preparation of standard working solution

Accurately weigh standard stock solution (A.3.2) of 0, 1, 2.5, 5, 10, 25mL into bottles of 25mL respectively and dilute them to the scale with primary water, and prepare them into the standard working solution of 0, 1, 2.5, 5, 10, 25mg / L respectively.

6. Pre-treatment of sample

6.1 Sample of raw material

Weight 100mg (precise to 0.01mg) sample for testing into a bottle of 100mL, dissolve it with primary water completely through ultrasonic treatment, make the volume constant, and filter the solution with 0.45um micropore filtering membrane for water system. The obtained filtrate is the solution of the sample for testing.

6.2 Sample of finished product

Weight about 50mg of the finished product powder or sample after grinding (precise to 0.01mg) and dissolve it into a bottle of 100mL with 50mL of primary water for 20min through ultrasonic treatment, and make the volume constant to the scale with primary water, filter the solution with 0.45um micropore filtering membrane for water system, move the filtrate into the sample bottle of liquid chromatography for testing. In case the sample testing concentration exceeds the working curve scope, test after diluting accordingly.

7. Chromatograph analysis conditions

7.1 Column temperature: 30℃.

7.2 Testing wave length: 254nm

7.3 Mobile phase A: Weight 13.6g potassium dihydrogen phosphate and 2.7g tetrabutylammonium hydrogen sulfate and dissolve them into 800mL of primary water, mix until they are completely dissolved. Use the potassium hydroxide solution to regulate the pH value of the solution to 3.5±0.05, supplement the solution with water to 1000mL, add 64mL of methanol, mix it evenly, leach with 0.45um micropore filtering membrane, and finally have ultrasonic degassing.

7.4 Mobile phase B: Use the measuring cylinder to weigh 700mL of methanol and make its volume constant at 1000mL with primary water, and keep it for later after ultrasonic treatment. The gradient elution program is as shown in Table 1

7.5 Sample volume: 10μL

Table 1. Gradient elution program

Time / min	Flowing speed / (mL/min)	B%
0	0.8	0
12	0.8	6
17	0.8	70
19	0.8	70
21	0.8	0
30	0.8	0

8. Sample testing

8.1 Sample of raw material

Under chromatograph conditions, test the standard stock solution (5.1) and raw material sample solution (6.1) respectively, record the area and preservation time of chromatograph peak. As per the area of the chromatograph peak, calculate the product content with area normalization method.

8.2 Sample of finished product

Under chromatograph conditions, test the standard working solution (5.3) and sample solution (6.2) respectively, record the area and preservation time of chromatograph peak. As per the area of the chromatograph peak, calculate the product content with external standard method.

9. Result calculation

9.1 Sample of raw material

9.1.1 The β-Nicotinamide Mononucleotide content in the sample is calculated per Formula (1)

$$P = \frac{A_T}{A_0} \times 100\% \quad \text{.....（1）}$$

In which, *P*—— the content of β-Nicotinamide Mononucleotide in the raw material, with the unit as percentage (%)。

A_T——Main peak area of β-Nicotinamide Mononucleotide in the standard stock solution

A_0——The area sum of all the peaks in sample solution of raw material.

9.1.2 The β-Nicotinamide Mononucleotide content in the sample is calculated per Formula (2)

$$X = \frac{C_T \times V}{\mathrm{m}} \times 10000 \quad \text{.....（2）}$$

Where: *X* -- in the test sample β- The content of nicotinamide mononucleotide was expressed in percentage (%);

C_T -- concentration of NMN in raw material sample solution (6.1) obtained by single point quantitative external standard method (mg / L);

V -- constant volume of sample (mL);

m -- mass of sample, in gram (g).

9.2 Sample of finished product

9.2.1 The net content of each finished product sample shall be calculated according to formula (3)

$$W = \frac{\mathrm{m\ total}}{N} \quad \text{.....（3）}$$

One capsule: W - net content of each finished product sample (g / capsule);

Mtotal -- weigh the total mass of all finished products (g)

N -- the quantity of all finished products (grains)

9.2.2 The β-Nicotinamide Mononucleotide content in the sample is calculated per Formula (4)

$$X=\frac{C\times V\times \mathrm{f}}{m\times 10000} \quad \text{.....（4）}$$

In which: X ——The content of β-Nicotinamide Mononucleotide in the sample of finished product, with the unit as percentage (%)

C ——β-Nicotinamide Mononucleotide concentration (mg/L) in the sample solution (6.2).

V -- constant volume of sample (mL);

f ——Dilution factor

m —— Weight of the sample of the finished product（g）.

Appendix A

(informative)

Figure 1 reference β- Liquid chromatography of nicotinamide mononucleotide